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EXPERIMENTAL STREPTOCOCCUS EMPYEMA

ATTEMPTS AT PREVENTION AND THERAPY BY MEANS OF VACCINES AND SERUM*

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An attempt was made in a recent survey of the literature to point out the great and growing importance of the streptococci in various human affections.[1] Emphasis was laid on the recently appreciated significance of hemolytic streptococci in bronchopneumonia and in the empyema which frequently follows it as it occurred in the early months of the late war in army camps. The importance of streptococcus empyema has not only not decreased, but has become intensified during the last year by its occurrence as a fatal sequel to influenza as well as in its previous relation to spontaneous bronchopneumonia and to bronchopneumonia after measles.

Our previous survey as to the actual accomplishment in the prevention and treatment of streptococcus infections by specific means, that is to say, by the use of vaccines and serum, gave us little encouragement. This study was undertaken not simply in an effort to proceed toward a possible practical method of treating streptococcus empyema, but in the desire to contribute some information to the larger question of streptococcus immunity as a whole.

THE GROUPS OF HEMOLYTIC STREPTOCOCCI

It was realized a few years ago that human pathogenic streptococci could be definitely divided by the blood plate method into hemolytic and nonhemolytic varieties. Of these two groups, the hemolytic organisms have been found associated with the more severe and generalized types of infection, such as septic sore throat, general septicemia, and the bronchopneumonia and empyema already mentioned. It was further known that the hemolytic streptococci may be divided

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* This study was begun while the senior author was in military service, and stationed at the laboratory of the Southern Department, Fort Sam Houston, Tex. It was continued at the Department of Research Medicine, University of Pennsylvania, and at the Yale Army Laboratory School, New Haven, Conn. The greater part of the experimental work was done in the Department of Pathology and Bacteriology, University of California. Thanks are due to a number of officers and enlisted men and assistants in civilian life, who under our direction have accomplished portions of the work described. In particular we wish to express our appreciation to Major J. S. Simmons and Lieut. W. C. Von Glahn, both of the Medical Corps, and to Lieut. J. J. Enright, and the late Paul D. Peltier, of the Sanitary Corps, to Sergt. F. Eberson, to Miss Dolores Bradley, and to Dr. Marjorie W. Cook.

[1] Gay, Frederick P.: Jour. of Lab. and Clin. Med., 1918, 3, p. 721.

on the basis of fermentation reactions in the sugars into some 8 or more groups (Holman). Of these latter it was estimated that only 2 or 3 varieties are numerically of great significance in human infections. There has seemed little evidence on which to associate any particular type of hemolytic streptococcus with any definite disease entity.

Until very recently the attempt to classify streptococci on an immunologic basis has simply indicated that the viridans, or the non-hemolytic group, in general is rather heterogeneous, whereas the hemolytic group seemed homogeneous. It is clear that the failure in preventive and therapeutic immunization against streptococci is due in large part to a failure to appreciate the diversity of the numerous types of organisms involved. Any progress is obviously dependent on the eventual recognition of the antigenic group varieties of the streptococci. In recent months, important contributions have been made toward the classification of the hemolytic streptococcus on immunologic lines by Hamilton and Havens,[2] Dochez, Avery and Lancefield,[3] and by Havens.[4] The work of Dochez, Avery and Lancefield in particular has brought out conclusively the fact that not only do such closely allied organisms as the hemolytic streptococcus pyogenes and the hemolytic Streptococcus infrequens (mannite fermenter) differ consistently and antigenically, but that Streptococcus hemolyticus itself contains three or more different immunologic types. Furthermore, these group varieties of Streptococcus pyogenes were isolated from the same group of empyema cases in the same locality (Fort Sam Houston). It is evident how markedly this subdivision of Streptococcus pyogenes in a given infection in the same locality will complicate any possibly successful vaccine or serum prevention, or treatment. by Dr. Avery, to the culture numbered 136 in their series, and as yet

PERSONAL INVESTIGATIONS

Our own work, although undertaken in ignorance of the subsequently recognized antigenic varieties in a supposedly single organism, was fortunately carried out with a single pure strain isolated from successive single colonies of a culture from one of the Fort Sam Houston cases by the Rockefeller Commission. This organism ("H") was obtained from the lung in a fatal case of bronchopneumonia complicated by empyema and pericarditis and corresponds, we are informed by Dr. Avery, to the culture numbered 136 in their series, and as yet

[2] Jour. Am. Med. Assn., 1919, 72, p. 272.
[3] Jour. Exper. Med., 1919, 30, p. 179.
[4] Jour. Infect. Dis., 1919, 15, p. 315.

unclassified. It belongs to Streptococcus pyogenes group, that is to say, it is hemolytic, and ferments lactose and salicin, but not mannite.

There is some suggestive evidence from previous work in streptococcus immunity that success which may have been obtained was due to a strict observance of the relationship between the infecting strain and the immune serum employed. There is, further, some suggestion that the best results have been obtained in human infections by using a recently isolated or "humanized strain" of the organism in producing the immune serum.[1]

In order to test the possibility of the latter hypothesis we have employed in our experimental work on rabbits, strains of our streptococcus grown on the one hand in a medium containing rabbit serum, and on the other hand, in a glucose medium. Throughout our work we have utilized beef infusion broth for the growth of the organisms adjusted to a reaction of P_H 7.2-7.4, and containing either 1% glucose or 5% sterile, fresh rabbit serum. The streptococcus grows readily on both these mediums, and in 24 hours presents a general diffuse cloudiness. When, however, a culture is transferred from serum to glucose broth it fails to grow rapidly in the first few subcultures. In 2 or 3 days the growth in the glucose broth, owing to the more rapid production of acid, becomes granular and sediments to the bottom of the tube, whereas the growth on rabbit serum broth remains turbid for a longer period of time. The serum cultures remain alive at 37 degrees for at least 2-3 weeks; glucose cultures are dead at body temperature in from 3-5 days. They may be preserved for 10-14 days, however, by growth at 37 or 24 degrees and subsequent conservations in the icebox.

In our work both on infection and immunity it was necessary to have a fairly accurate enumeration of the number of bacteria contained in a cubic centimeter of either of these cultures. We had been somewhat skeptical of the strict accuracy of the Wright method of counting bacteria by comparison with red blood cells, but a careful check of the method in a number of instances by gravimetric tests has convinced us of its unusual accuracy. Counts made at intervals during the progress of the work by Wright's method have given very consistent results. The average of 26 counts of 24 hour H. R. S. cultures (by which is meant the "H" strain used for our work grown on rabbit serum broth) gave 1,207 million cocci to the cubic centimeter. Glucose cultures gave an average of 750 million per c c. The chains of organisms grown on the glucose medium are longer than those grown on the serum medium,

and the individual cocci appear swollen and of greater diameter. As already stated, efforts have been made to check the accuracy of this simple method of enumeration of bacteria. The plating method, which is so successful in checking counts of this sort when carried out with organisms like the typhoid bacillus, appears to be quite valueless with the streptococcus. It has been found that succesful dilution beyond a certain point may continue to show as many or even more organisms than the next supposedly lower dilution in the series. It may be that this increasing count is due to the mechanical breaking up of the coccal chains. At all events, it was found necessary to abandon the plating method after numerous attempts; a gravimetric method was next tested, and found to show a striking correspondence of weight of dried streptococci as compared with the Wright method.

In table 1 are given several Wright counts as compared with the dried weight of the bacteria derived from 50 c c of the same culture from which the count had been made. The organisms were collected in the bottom of a tube by prolonged centrifugalization until the supernatant culture fluid was perfectly clear. After pouring off the supernatant fluid the sedimented culture was shaken in 10 c c of absolute alcohol, recentrifugalized, shaken and centrifuged again in 10 c c of absolute ether. A constant dried weight of baceria was obtained in 2 or 3 days by placing the tube in a partial vacuum over calcium chlorid.

TABLE 1

WRIGHT COUNTS AS COMPARED WITH DRIED WEIGHT OF BACTERIA

Culture	Wright's Count in Millions per c c	Milligrams of Bacteria in 50 c c	Millions of Bacteria per Milligram
H. R. S. 35*	459	6.8	3,381
H. R. S. 36	425	6.3	3.377
H. R. S. 41	619	8.1	3,821
H. R. S. 42	450	6.1	3,689
			Aver. 3,592
H. D. 27†	508	3.4	7,470
H. D. 32	833	5.3	7.871
H. D. 28	600	5.5	5,454
H. D. 36	760	5.6	6,800
			Aver. 6,898

* The serum broth employed of this serum was of slightly different composition which accounts for the lower counts as compared with the average found in later experiments.

† Cultures o f "H" on 1% glucose broth, with generation of culture.

An inspection of the table brings out not only the close correspondence between the direct counts of bacteria and their weight, but shows another interesting fact worthy of further consideration at some time, bearing on the metabolism of bacteria in culture media of different constitution. It will be noted that although in this particular series the counts of bacteria in the glucose cultures ran somewhat higher than those in the serum cultures, the weight of the organisms themselves was distinctly less when grown on glucose than when grown on serum. This observation should be still further correlated with the fact that the organisms in glucose cultures are distinctly larger than when grown in serum mediums. As a further check on the accuracy of the comparative estimates in these experiments, it should be stated that Wilson and Dickson[5] in their article on a "Gravimetric Method of Standardizing Vaccines" found that Streptococcus fecalis grown on agar contains 3,398 million streptococci to the milligram, which is in close correspondence with our averages.

EXPERIMENTAL EMPYEMA IN RABBITS

Our culture "H" of Streptococcus pyogenes agrees with the characteristics described by other investigators for organisms of this group. It is not markedly pathogenic for rabbits. Dochez, Avery and Lancefield found that 1 c c or more of a 24-hour broth culture administered intraperitoneally was necessary to kill rabbits. Davis[6] found that pathogenic human strains of the hemolytic streptococcus will rarely kill rabbits in a dose of less than 0.5 c c of a broth culture per kilogram of a rabbit, and the most pathogenic strains are apparently less fatal for these animals than the other strains. Our culture "H" soon after isolation would at times kill rabbits in from 2-4 days in a dose of approximately 0.5 to 1.0 c c per kilo administered intravenously. In successive subcultures on rabbit serum broth it became less pathogenic for rabbits, even when strains of the original "H" organisms that have been passed through the pleural cavity of rabbits for a number of generations are employed as the inoculum for broth cultures. When death does occur in these animals, the organism is found in the heart's blood, and in many instances produces a localized arthritis. An attempt to increase the general invasive properties of this culture, "H," for rabbits has been unsuccessful. It apparently cannot readily be induced to produce a general septicemia with uniformity.

[5] Jour. of. Hygiene, 1912, 12, p. 49.
[6] Jour. Am. Med. Assn., 1919, 72, p. 319.

We have, however, succeeded with great regularity in producing a streptococcus pleuritis or empyema in rabbits which we believe resembles in all respects its human analogue. In our early experiments we succeeded by the use of relatively large amounts of a culture of the "H" organism in producing pleuritis in a few rabbits, although somewhat inconstantly. When 1-3 c c of a 24-hour culture of "H" in its early generation on rabbit serum or blood broth was injected into the right pleural cavity or rabbits, 7 out of 10 animals showed at death, or when killed within a week, various stages of an extending pleurisy.

At this point it may be well to review our present conception of the pathogenesis of human empyema due to streptococci. It is clear from the observations of numerous investigators that the hemolytic streptococcus is present in the throat of from 20-50% of normal men in army camps. It has been found by Pilot and Davis[7] in 90% of the crypts in follicular tonsillitis, where it apparently serves as a potential focus for further invasion. It has further been evidenced by numerous observers since the work of Cole and MacCallum[8] that patients with measles who harbor these organisms in their throats are much more likely to suffer from the unfortunate sequels of bronchopneumonia and empyema than patients with "clean" measles. Streptococcus bronchopneumonia, whether or not complicated by empyema, has been found to occur in otherwise healthy individuals as well as a sequel to measles, scarlet fever and influenza. The bronchopneumonia frequently represents a transitory phase in the extension of the streptococcus from the nose and throat through the lungs to the pleura. There seems, further, little reason to question that this organism spreads by a process of direct extension rather than by metastasis. The extension may be extremely rapid as is evidenced by those instances of empyema which occur in individuals that were apparently well and working a day or two before. Bronchopneumonia is frequently not diagnosed if present until the occurrence of empyema. Blood cultures taken from human cases during the course of bronchopneumonia and empyema are almost always sterile except in the terminal phases of the disease (MacCallum[9]). Human streptococcus empyema in numerous cases continues its invasion by extension by involving first the pericardium and then the pleural cavity on the

[7] Jour. Infect. Dis., 1919, 24, p. 386.

[8] Jour. Am. Med. Assn., 1918, 70, p. 1146.

[9] The Pathology of the Pneumonia in the U. S. Army Camps During the Winter of 1917-18, Monograph No. 10, Rockefeller Institute for Medical Research, 1919.

opposite side. Metastatic complications are extremely rare; the process as a whole may be classified as infection by extension.

The experimental empyema in rabbits fulfills in every detail the human analogue as we have outlined it. We have not, however, succeeded in producing streptococcus bronchopneumonia in rabbits. It will be recalled in this connection that Lamar and Meltzer produced a typical lobar pneumonia in dogs by bronchial insufflation of cultures of pneumococcus. Winternitz and Hirschfelder[10] produced similar results with the same organism in rabbits. Wollstein and Meltzer[11] claim to have produced streptococcus bronchopneumonia in dogs, but so far as we are aware, no attempt has been made heretofore to produce it in rabbits. At all events, we have not succeeded in so doing, although we have insufflated large amounts (5-10 c c) of a culture into the bronchi through a rubber catheter inserted in the trachea, followed by a syringe full of air. The animals when killed in one or two days had normal lungs. In a few instances we tried to obtain results in rabbits that had been previously gassed with chlorpicrin. The three animals thus tested died under the anesthesia before the culture could be administered.

EXPERIMENTAL PROCEDURE

We have not, therefore, succeeded in producing the entire course of a human streptococcus empyema in rabbits. Empyema may be regularly produced, however, in rabbits by this streptococcus by direct injection of small amounts of culture into the pleura, particularly when the strain employed has been passed through the pleura of several rabbits. Small amounts of the culture, as will be discussed in detail, are injected in a volume of 1 c c through a buttonhole in the skin on the right side of the rabbit without anesthesia. The skin is painted with iodin and the fluid injected into the right pleural cavity, which is easily reached by pushing the needle gently through the muscles lying over the second and third interspace to a point about half an inch to the right of the sternum. Errors in technic which may occur in the first few animals are easily detected at necropsy by the occurrence of an abscess in the muscles on the one hand, or by an abscess in the lung when the needle has been pushed too far.

As already mentioned, even large doses (1-3 c c) of early "H" cultures grown on rabbit blood broth or rabbit serum broth did not uniformly produce a pleurisy in rabbits. Attempts were then made to produce pleurisy by injecting the pleural fluid from one animal, containing variable numbers of streptococci, into a second animal. These results were likewise inconstant. When subcultures were made from the pleural fluid of an animal that had suffered from fatal empyema, constant results were obtained, particularly if the fluid represented a strain that had been passed through the pleura of several (4 or more) animals. The pleural fluid from an infected rabbit can be preserved in a

[10] Jour. Exper. Med., 1913, 18, p. 657.
[11] Ibid., 1913, 16, p. 126.

sterile tube in the icebox for several weeks and used in numerous experiments. It has been found that the organisms in this pus remain alive for a long period of time. Twenty-four-hour cultures grown on 5% rabbit serum broth from a passage pleural fluid have therefore been used in numerous experiments. Such cultures in proper dosage produce a constant and characteristic extending empyema when injected into the pleural cavity of normal rabbits. The minimum infecting dose of a passage culture, as grown directly from pleural fluid for 24 hours in rabbit serum broth, is 0.1 or 0.2 c c. Thus, we find that of 16 normal rabbits that received intrapleural injections of 0.1 c c of passage pleural culture, 14, or 87%, showed a characteristic pleurisy. Of 14 animals given 0.2 c c of a 24-hour culture intrapleurally, all, or 100%, were positive.

The course of the pleuritis produced in rabbits by this means may properly be described as subacute, that is to say, it is sufficiently prolonged to resemble in some respects the human disease and to offer sufficient time for any therapeutic intervention that might avail. Death occurs in from 1-17 days in the numerous instances in which we have observed it. On an average it has occurred on the fifth day. There is no evidence that spontaneous recovery takes place, at least not in our later series in which a uniform passage culture in a constant dose was employed. The animals show progressive loss of weight, and if the disease is prolonged considerably, respiratory distress follows until death intervenes. Having determined that death is the invariable result of this infection, it has been found more expeditious in most experiments to kill all surviving animals in from a week to 10 days and to note their condition of recovery or advanced pleuritis, rather than to wait for death to intervene. This procedure has not only made a greater number of experiments possible, but has given information as to the destruction of the organisms during the course of the pleurisy.

As in human cases it was found that pleurisy usually spreads rapidly by extension, and involves the pericardium and the other chest cavity. Thus, of 103 animals examined, 47 (45%) were found to have both chest cavities and the pericardium involved, 23 (22%) were localized in one side alone, 17 (16%) involved both pleural cavities, and 15 involved one chest cavity and the pericardium. In a single instance the pericardium alone was involved, possibly through injection into this sac. Extension may be very rapid as was evidenced in a number of animals that died in 24 hours in which both pleural cavities and the pericardium were found involved. Mediastinal abscesses filled with thick yellowish pus are frequently found adjacent to the lower anterior margin of the lungs, as described by MacCallum[9] in the human cases.

The nature of the exudate likewise resembles in its appearance at successive periods the human empyema fluids as MacCallum has described them, except that it is never greenish in color. In earlier cases it is serous in nature, and in 2 or 3 days becomes seropurulent, then fibrinous or fibrinopurulent. As much as 15 or 20 c c of fluid may be found in one chest cavity. On examination the fluid contains large numbers of leukocytes and usually, but not always, numerous streptococci are seen in chains of varying length. The cultures from the pleural fluids irrespective of their appearance are always positive. The lung on the affected side is collapsed, atelectatic and covered with a thick matwork of fibrin, although it may be insufflated artificially as in the human cases described by MacCallum.

The leukocytic counts from the peripheral blood do not vary much from those in normal rabbits for several days, but when followed they show a sharp rise to from 20,000 to 30,000 two or three days before death. In one case, complicated by a broken leg, there was an immediate leukocytic increase and the formation of pus in the region involved. In animals that are killed in from 5-10 days while suffering from pleurisy, only 24% in a total of 41 have shown the presence of streptococci in the circulating blood, even when infecting doses as large as 1 c c are employed. In animals that are allowed to die spontaneously the blood cultures are more frequently positive, although even in these cases they were found in only 69% of 23 animals examined from this standpoint. There is evidence then that septicemia as in human beings is not characteristic, but that we are dealing rather with an infection by extension.

It should be clearly emphasized that there is no evidence in our own work to indicate an elective localizing affinity in the sense of Rosenow.[12] We tried, without success, in the early stages of our work, to obtain localization in the pleural cavities by intravenous injection, and in many attempts since subcultures from an empyema fluid given intravenously in amounts as large as 10 c c have failed in all cases to show localization in the pleura. Several cubic centimeters of the pleural fluid itself injected into the veins also failed to produce pleuritis.

ACTIVE IMMUNIZATION AGAINST EXPERIMENTAL EMPYEMA

We have seen in our previous review[1] that there is little evidence as to the effectiveness of active immunity in animals produced by

[12] Jour. Am. Med. Assn., 1915, 65, p. 1687.

injections of streptococcus vaccine. There was, until recently, no information as to the possibilities of systematic vaccine prophylaxis against this organism in human beings. In recent months several communications bearing directly or indirectly on this problem have appeared. Ely, Lloyd, Hitchcock, and Nickson[13] have attempted systematic immunization against streptococcus infections in the Puget Sound Navy Yard. They found hemolytic streptococci in the majority of their fatal cases of influenza, and after immunization of considerable numbers of men with these organisms they have apparently noted lessened incidence of influenza in the vaccinated as compared with the unvaccinated men, and a very striking decrease in the mortality in the former category. There were actually no deaths in over 4,000 vaccinated men, whereas deaths varied in from 4-12% of the cases of influenza that occurred among the unvaccinated. These authors do not speak of the instances of complications in either group, nor do they mention the existence of empyema. Rosenow, who believes a diplostreptococcus of the viridans type bears a causative relation to influenza, has advocated an immunization with a mixed vaccine containing those organisms present in the throats of influenza cases and including 30% of the viridans streptococci found there. His results indicate a lessened incidence of influenza in the vaccinated and a reduction in some of the complications, notably in the pneumonia which is only one third as frequent in the vaccinated as in the unvaccinated. No note is made as to the occurrence of empyema. Richey,[14] who was evidently dealing with a mixed vaccine similar to Rosenow's,[15] found that experimental tonsillitis was produced accidentally in a number of individuals who were given nasopharyngeal washings and bronchial secretions from cases of influenza in an attempt to produce the latter disease experimentally. This tonsillitis was found to be due to the hemolytic streptococcus present in such washings. He notes that "this tonsillitis occurred in three individuals who had received one month previously a vaccine containing, in addition to other organisms, three presumably adequate doses of haemolytic streptococci."

There is then no evidence to lead us to anticipate that either generalized or localized infections due to streptococci are prevented in human beings by the ordinary methods of vaccination which have been so successful in certain other bacterial infections.

[13] Jour. Am. Med. Assn., 1919, 72, p. 24.
[14] Jour. Infect. Dis., 1919, 25, p. 305.
[15] Jour. Am. Med. Assn., 1919, 72, p. 31 and p. 1604.

Rabbits may be treated with repeated injections of either killed or living "H" culture owing to the fact that it is not markedly pathogenic for these animals. An extensive series of animals has been treated in this manner not only in order to test their active protection against the pleurisy described, but also to test the properties of their serums in treating this infection. The animals were immunized, not only with serum broth cultures of streptococcus, but also in a few instances with glucose cultures in order to determine any possible influence of the immediate source of the organisms used in preparing the vaccine. Immunizations were usually carried out by gradually increasing the dose first of killed and then of living culture given at varying intervals and for periods extending for from one month to two and a half months. In some instances only living cultures were employed beginning with small doses. The animals lost weight during the course of immunization, but usually regained it if the series of inoculations was interrupted for a short time. A week or ten days after the last injection they were given intrapleural inoculations of the test dose of a subculture from the pleural fluid in the manner described. It will be recalled that 0.1 c c of a 24-hour serum broth culture almost invariably produces a progressive pleuritis and pericarditis (87% positive). In all instances tried 0.2 of a c c of a 24-hour culture has invariablly produced this syndrome. We are not only thus assured by the continued repetition of this experiment that it may be produced invariably in normal animals, but in each instance where two or more of the immunized animals to be tested were inoculated one or more control normal animals were run through at the same time. In every instance included in the following table the normal controls succumbed to the characteristic infection.

In table 2 are given the results obtained in producing an experimental empyema in rabbits previously immunized as outlined. They are arranged in order of the total amount of bacteria used in immunization. In the table is included, first the type of vaccine employed in immunization, whether obtained by growing the culture "H" on rabbit serum broth (vaccine "S") or on glucose broth (vaccine "D"). There is further mention here as to whether the animals received killed cultures alone or living cultures alone, or killed and living cultures. The number of injections and the total number of organisms used in the entire process of immunization, computed in billions, is then added. In the next column the infecting pleural dose is given and represents the amount of a 24-hour culture in serum broth from the pleural fluid of a rabbit previously dead of empyema. The time of death ("+") or killing ("X") is noted and the results found as regards the presence of pleurisy, and whether the latter is single or double (+ or + +), and also the occurrence of pericarditis.

TABLE 2

EXPERIMENTAL EMPYEMA IN IMMUNIZED RABBITS

No.	Vaccine	Injections	Total in Billions	Infecting Dose, c c	Killed or Dead	Pleurisy	Pericarditis
243	S killed	9	2¼	0.2	+ 7	++	+
244	S killed	9	2¼	0.2	+ 5	++	+
245	S killed	9	2¼	0.2	x 5	++	+
223	S killed and living	6	3	0.1	x 5	0	0
220	S killed	6	3	0.2	x 6	0	0
246	S killed	12	3¾	0.1	+ 4	++	+
251	S killed	14	4¾	0.1	x 6	0	0
229	S killed	9	6	0.1	+ 5	++	+
232	S killed	9	6	0.1	x 6	0	0
234	S killed	10	6	0.1	x 5	0	0
532	S living	9	6	0.2	x12	+	0
534	S living	9	6	0.2	x11	0	0
302	S killed and living	12	9	0.1	x 6	0	0
303	S killed and living	13	10¾	0.1	x 4	0	0
305	D killed and living	11	14	0.1	x 4	0	0
306	D killed and living	12	15	0.1	x 6	0	0
257	S living	15	26	0.2	x10	0	0

An inspection of this table shows clearly that a considerable number of injections of killed cultures can be used without producing protection against the experimental pleurisy and that killed cultures followed by living cultures must be given for a long period of time and in large aggregate amounts in order to assure complete active immunity against the localized infection. It is evident that if sufficient amounts of killed and subsequently of living cultures of streptococci are given over a considerable period of time, representing a large number of injections, protection against empyema is assured. The total number of bacteria employed in immunization rather than the number of injections given would seem to be the decisive factor. This would agree with the results attained in typhoid vaccination. There is apparently as good protection afforded by cultures of organisms grown on glucose as when they are grown on the serum of the animal in which protection is induced.

It seems evident that no optimistic conclusion can be drawn from these results in rabbits as to the possibility of protecting human beings against localized streptococcus infections, or specifically against empyema, owing to the large amount of vaccine and prolonged nature of the treatment required.

ATTEMPTS AT SPECIFIC THERAPEUTIC INTERVENTION IN EXPERIMENTAL EMPYEMA

Attempts to cure or prevent streptococcus infections by immune serum have been made almost entirely in generalized infections. This

is true not only of the earliest work of Marmorek,[16] but also in the recent work of Dochez, Avery and Lancefield. We do not recall instances in which streptococcus immune serum has been utilized in treating local streptococcus infections either experimentally in animals or in human beings. Our results then are of interest not only as attempts to cure experimental streptococcus empyema, but as possible lines of attack on other localized infections due to this organism.

We have already recounted in some detail the methods employed in immunizing rabbits against our particular strain of streptococcus. Both glucose broth and serum broth cultures have been employed and the immunization was continued over a considerable period of time in varying doses, but, in all the completely protected animals, with large total amounts of living bacteria. As we have just described in the preceding section, we have succeeded by prolonged treatment in producing a very definite active immunity against intrapleural infections with this streptococcus. The animals that were tested for active immunity were killed by bleeding from 7-12 days after the trial intrapleural injection designed to test their protection and their serum employed in the curative experiments that follow. It is evident from the fact that they are obtained from actively immunized animals that they are, properly speaking, immune sera. In addition, we have tested the content of antibodies in these sera to determine the presence of antagonistic properties to the streptococcus used for immunization. Unfortunately, we have not hitherto been able to produce a constant septicemia in rabbits or mice with our strain "H" by which we could have tested the protective or curative properties of such serums in preventing generalized infections. The tests for antibodies employed have been principally for agglutinins, opsonins and precipitins, all of which have been positive in high degree.

Agglutinin Tests.—Much difficulty in the past has been found in demonstrating agglutinins in the serum of individuals suffering from streptococcus infections or in animals immunized against streptococci. The technical difficulties have been so great that many have been unable to satisfy themselves that agglutinins really are present under these conditions. In the light of our present knowledge, it would seem that failure to demonstrate agglutinins to streptococci is, however, a purely technical matter. The great difficulty has lain in obtaining cultures of streptococcus that are not spontaneously sedimented or flocculated, and various measures, such as mechanical attempts to

[16] Ann. de l'Inst. Pasteur, 1895, 9, p. 593.

break up the chains and the addition of alkali, have been tried to obviate this difficulty. Although we have succeeded in establishing a wholly satisfactory technic for agglutination tests of streptococci, it was done at first without appreciation of the mechanism involved, which has since become clear. It is desirable from every standpoint to have a standardized homogeneous suspension of organisms which can be used for considerable periods of time in making agglutination tests, and our efforts to obtain such suspensions were at first unavailing. We found finally, however, that a constant homogeneous suspension of streptococci may be obtained by adding phenol in a final dilution of 0.2 to 0.5% to a 24-hour serum broth culture. The cultures so treated are found sterile on the following day, after incubation at 37 degrees, and may then be concentrated by centrifugalization and resuspended in the same serum broth in which they have been grown in one-tenth the amount of the original fluid. This gives a thick suspension of the organisms which does not sediment except over a period of several days. The fact that complete and rapid sedimentation resulted when we first centrifuged the cultures and then suspended them in 0.5% phenolated salt solution led to the explanation of the success or the failure of these slight variations in technic. If phenol is added to carefully neutralized physiologic salt solution of a P_H of 7.2, the eventual 0.5% solution becomes approximately of a P_H of 6.9. If, however, the same amount of phenol is added to serum broth, it does not change more than to a P_H of 7.1. This slight difference in acidity then would seem to be the important factor in spontaneous sedimentation of these bacteria. We suspect, moreover, that it may have a more far reaching significance in those cultures described as "spontaneously" agglutinable. Physiologic salt and indeed distilled water, as prepared in the ordinary laboratory, is frequently not exactly at the neutral point, and very slight deviations in the acidity may give a flocculation of the type we have encountered in streptococci. Our work in preparing the antigen agrees completely with that of Dochez, Avery and Lancefield, and we further agree with them in obtaining far better agglutination results by incubating the agglutination tests at 55 C. instead of at 37. The results are more rapidly obtained, and the final reading is in higher dilution at the higher temperature. Controls with normal serum by this technic with the phenolated suspension of streptococci are uniformly negative even in low dilutions.

The immune serums we have produced gave positive agglutination reactions at 55 C. in dilutions of from 1:400 to 1:12,800. There is

apparently no difference in the results of agglutination when the suspension of streptococci has been grown on glucose mediums, or when the serum of animals immunized by glucose cultures is employed with serum culture suspensions. Cultures of other streptococci of the pyogenes group obtained from the same group of empyema cases at Fort Sam Houston either gave slight agglutination or none at all with our antiserum to strain "H." This organism, it will be recalled, remains unclassified in the Dochez, Avery and Lancefield grouping, and our results, although largely negative for other strains, suggest findings similar to theirs.

Immune Opsonin Tests.—A 24-hour culture grown in serum broth was used. The culture was counted and diluted with broth, so that each cubic centimeter contained 500,000,000 organisms.

Leukocytes were obtained from the peritoneal fluid of a guinea-pig which had been injected intraperitoneally 24 hours previously with 10 c c of sterile broth. The peritoneal exudate was washed 3 times in 0.9% NaCl and the final sediment suspended in 0.9% NaCl.

The serum was inactivated for half an hour at 56 C., and used both undiluted and in dilutions of 1:50, 1:100, 1:200, 1:400, etc. In a control test with normal serum lower dilutions of 1:20, 1:40 and 1:80 were used.

An immune serum of known titer was included in each test to furnish a basis of comparison for the activity of each preparation of leukocytes.

In the case of the immune and normal sera, 0.1 c c each of serum dilution, culture, and leukocytes were mixed in small test tubes. As a control on the phagocytic activity of the leukocytes alone, the serum dilution was replaced by 0.9% NaCl, the tube containing only leukocytes, culture, and salt solution. All tubes were incubated for 2 hours at 37 C., the tubes being shaken frequently during the first hour.

At the end of the period of incubation, the supernatant fluid was poured off from each tube, leaving only a drop or two of fluid, in which the sedimented bacteria and leukocytes were thoroughly mixed. From this mixture smears were made with a loop on slides. The smears were fixed in picric acid and stained with alcoholic eosin and methylene blue.

Estimation of the opsonic properties of the immune serum was made as follows:

With each slide a count was made of the number of leukocytes which contained streptococci in a total of 100 cells. Counts of the leukocyte control tube gave the lowest percentage — about 12%. The dilution of serum which gave results comparable to the leukocyte control tube was considered the point of bacteriotropic extinction. In the case of normal serum this point was reached usually in a dilution of 1:40, rarely of 1:80. With the immune serums much higher dilutions, sometimes as high as 1:3,200, were required. The titer of the serum was considered to be the last dilution in which there was distinct evidence of phagocytosis above that shown in the leukocyte control. For a normal serum, as indicated above, this value was usually 1:20; for an immune serum anywhere from 1:200 to 1:1,600. By means of these figures, the activity of an immune serum was readily expressed as the multiple of the activity of a normal serum — an immune serum of 1:1,600 titer, for instance, being 80 times as active as a normal serum of 1:20 titer.

The serum of our animals in which active immunity had been proved by intrapleural inoculation was found to vary in tropin content from 5 to 80 times that of normal rabbit serum.

Precipitin Tests.—We have not obtained as good results in precipitin tests by the method described by Barnes[17] in this department who was working under somewhat different conditions with the larger group relations of streptococci. He employed the supernatant fluids of broth cultures of streptococci as antigen. We have, however, obtained constant precipitin results in our strictly specific relations as regards organisms and antiserum by employing extracts of dried "H" streptococci grown either in glucose or serum broth and ground in an agate mortar (operated by a mechanical ore grinder run by a small motor, for about one hour) until reduced to an impalpable powder. The immune serums employed by us for therapeutic purposes gave a marked precipitin reaction with these extracts of streptococci. The results were obtained by adding 0.5% carbolated salt containing an extract of from 1-10 mg. of dried ground culture to equal amounts of immune serum, both volumes used being 0.5 c c. No result was obtained with normal rabbit serum, even with the largest amounts of antigen employed. The results were usually positive at 37 C. by the ring test in from 15-30 minutes, but the ingredients were ordinarily mixed and allowed to stand over night in the incubator, and the final results read by sedimentation in the morning.

In general, the opsonin, agglutinin, and precipitin titers of our immune serum were found parallel, and in correspondence, so far as our figures go, with active protection, as illustrated by the following selected cases:

TABLE 3

RESULTS OF PRECIPITIN TESTS

Rabbit No.	Active Immunity	Agglutinins	Opsonin	Precipitins with Milligrams of Antigen
532	Negative	200	0	5
249	Not tested	200	25	5
304	Not tested	800	2.5	5
305	Positive	12,800	40	5
306	Positive	1,600	20	5
301	Not tested	6,400	80	1
251	Positive	12,800	20	—

Although we have made numerous attempts to demonstrate bactericidal properties in our immune sera after the manner described by Haven,[4] we have not succeeded in doing so. It should be remarked,

[17] Jour. Infect. Dis., 1919, 25, p. 47.

moreover, that our sera as measured by the agglutinin test were of greater potency than those employed by Havens.

Our antistreptococcus serum when mixed with a suspension of rabbit blood cells and living streptococcus culture has distinct anti-hemotoxic properties. The red blood cells of an immunized rabbit appear to be no more resistant to hemolysis than those of normal animals.

PREVENTION AND THERAPY OF EXPERIMENTAL EMPYEMA BY IMMUNE SERUM

A number of these immune sera produced in the manner described and containing strong antibodies have been tested for their preventive and curative action on experimental streptococcus empyema in rabbits. The injections of serum have been given before the intrapleural infecting dose, simultaneously with the dose, and at periods subsequent to it. One or two doses of 0.5 to 1 c c have been given, for the most part, into the infected pleural cavity, but in some instances intravenously. In each separate experiment results obtained by immune serum have been controlled by rabbits infected at the same time, but treated with normal rabbit serum.

Our results are distinctive, and indicate that experimental empyema may, in a definite though small percentage of cases, be prevented or even cured by the use of immune serum. They may best be summarized and then illustrated by an experimental protocol:

The immune serums from 10 different rabbits have been tested, most of them chosen as containing the highest content in antibodies. In 8 of the 10 rabbits furnishing this immune serum the active immunity against experimental empyema was tested and found to be complete. The other two were not tested. To repeat and add to the animals used as controls, it may be stated:

(1) That 14 normal rabbits inoculated with 0.2 c c of a 24-hour passage pleural culture of streptococcus "H" showed in every instance a positive empyema.

(2) 15 normal rabbits inoculated in the same manner and treated with normal rabbit serum in doses corresponding in amount and with the same routes of inoculation employed in the rabbits treated with immune serum, were also in every instance positive.

(3) In contrast to these 29 animals giving positive empyema in 100% of instances, we have succeeded in preventing or curing pleurisy by the use of rabbit immune serum in 5 of 20 animals when the

immune serum was given into the pleural cavity that contained the infecting dose, and in 1 of 3 animals in which the immune serum was given intravenously. Of the 5 animals in which the evolution of experimental pleurisy was prevented by the use of immune serum intrapleurally only two were, strictly speaking, cured, because in three instances 1 c c of serum was injected simultaneously with the culture and the treatment must be regarded as preventive. In the other two cured animals 1 c c of immune serum was in one instance given 24 hours after injection of the infecting dose, and in the other instance 0.5 c c of immune serum was given 6 and 24 hours after infection.

As an example of the experimental results obtained the full protocol of an experiment is herewith included:

Exper. 1.—A series of rabbits was given 0.2 c c of a 24-hour serum broth culture of passage pleural strain of streptococcus "H" intrapleurally. They were then treated with immune serum No. 305 or normal serum as follows:

TABLE 4

RESULTS OF IMMUNE SERUM TEST

No.	Serum Given	Death	Pleurisy	Pericarditis	Pleural Culture
584	1 c c Serum No. 305 simultaneously intrapleurally 1 c c 24 hours later intrapleurally	Killed 11 days	0	0	0
581	1 c c Serum No. 305 simultaneously intrapleurally 1 c c Serum No. 305 24 hours later intrapleurally	Killed 5 days	0	0	0
585	1 c c Serum No. 305 24 hours after culture intrapleurally	Killed 9 days	?*	0	+
586	1 c c Serum No. 305 24 hours after culture intrapleurally	Dead 2 days	++	+	+
583	1 c c Serum No. 305 simultaneously intravenously 1 c c Serum No. 305 24 hours later intravenously	Dead 4 days	++	+	+
587	1 c c normal serum simultaneously intrapleurally 1 c c normal serum 24 hours later intrapleurally	Dead 2 days	++	+	+

* The right pleural cavity showed firm fibrous adhesions between the partially collapsed lung and the parietal pleura. A very slight amount of bloody fluid was also present. The left pleural cavity and pericardium were normal. This finding is in sharp contrast to the double empyema usually found (++) and would indicate a healing process.

It is evident, then, that although distinct results in the prevention of experimental empyema and, in rare instances, the cure of experimental empyema, may be produced by the use of immune serum from rabbits, we have as yet no evidence of a sovereign or even encouraging serum therapy to offer. It would seem that the immune sera that

we have employed, to judge from their antibody content, are not only as potent as those used by other investigators, but they would appear, moreover, to reach the limits of potency ordinarily to be obtained.

ATTEMPTS AT VACCINE THERAPY

In several animals an attempt has been made to influence the course of the experimental empyema we have described by giving injections to kill streptococcus culture intravenously, such injections beginning either on the day of the intrapleural inoculation or actually preceding it by 24 hours. In certain of these vaccinated animals the leukocyte counts reached very high figures—in one instance 64,000 to the cubic millimeter, and distinctly exceeded the counts made in control, unvaccinated, infected animals. In no instance, however, was the slightest effect produced on the evolution of the empyema. The vaccinated, infected animals died, not only as rapidly, but apparently even more rapidly than the unvaccinated controls.

A combination of vaccine and serum therapy seems worthy of trial, and our results in this respect may subsequently be given.

SUMMARY AND CONCLUSIONS

This experimental study was undertaken with the practical problem of human streptococcus empyema in mind. It may be regarded as bearing on the general question of streptococcus immunity, and particularly as indicating the possibilities of specific prevention or therapy of localized streptococcus infections of the empyema type.

Human streptococcus empyema may be regarded as an infection by extension in contradistinction to generalized streptococcus septicemia. From the beginning our work was carried out with a single pure strain of the streptococcus isolated from a case of human empyema, and thus we have inadvertently avoided the errors that have beset other workers in this field in view of the recently discovered fact that not only hemolytic streptococci in general but Streptococcus pyogenes in particular is divisible into several distinct immunologic types. This study has dealt with a strictly homologous infecting organism and corresponding immune principles. Still further possibilities of specific correspondence have been investigated by comparing the results from growing the organisms on the serum of the animal to be infected, in comparison with growth on media containing glucose. No differences in the antigenic property of the organisms grown on these two media, have been found.

Although the human strain of streptococcus employed in this study is not markedly pathogenic for rabbits, it has been found possible to produce a constant, characteristic, and fatal extending pleurisy or empyema in these animals by the use of a passage culture of the original strain carried through the pleural cavity of several animals. This empyema is produced by direct injection of a small amount of passage culture (0.1 to 0.2 c c) into the pleural cavity. Injections of large amounts of such a passage culture or of the original empyema fluid from which it is derived, are not fatal when given intravenously and the organisms do not show selective localization in the pleura. This experimental empyema resembles in appearance the human analogue, which it was aimed to simulate, except that the initial bronchopneumonia which may occur in the human cases has not been produced in rabbits. It is a streptococcus infection by extension and very rarely becomes generalized before death. It is invariably fatal, on an average in 5 days, thus allowing sufficient time for therapeutic intervention.

Experimental streptococcus empyema in rabbits may be prevented by previous immunization with killed, followed by living, cultures of the same strain of streptococcus, but only when repeated vaccinations have been practiced and in a total amount which would seem to preclude the practicability of such a preventive inoculation in human beings, provided the conditions are similar.

The serum of rabbits that have been successfully immunized against subsequent intrapleural infection with the streptococcus contains potent antibodies, particularly agglutinins, opsonins, and precipitins. As the technic of the determination of such antibodies in antistreptococcus serum has been questioned, it is herewith fully detailed.

The more potent of these antisera from rabbits when injected simultaneously with the culture into the pleural cavity may prevent the evolution of the otherwise invariable and fatal empyema. In a few instances subsequent injection of serum into the pleural cavity, or even intravenously, has cured the empyema.

No very optimistic conclusion on the practical use of an antistreptococcus serum in this type of localized infection can be drawn from our experiments as hitherto conducted.

Attempted vaccine therapy of the localized empyema has given consistently negative results.